Microtubules Modulate the Stiffness of Cardiomyocytes Against Shear Stress

Satoshi Nishimura, Shinya Nagai, Masayoshi Katoh, Hiroshi Yamashita, Yasutake Saeki, Jun-ichi Okada, Toshiaki Hisada, Ryozo Nagai, Seiryo Sugiura

Abstract—Although microtubules are involved in various pathological conditions of the heart including hypertrophy and congestive heart failure, the mechanical role of microtubules in cardiomyocytes under such conditions is not well understood. In the present study, we measured multiple aspects of the mechanical properties of single cardiomyocytes, including tensile stiffness, transverse (indentation) stiffness, and shear stiffness in both transverse and longitudinal planes using carbon fiber–based systems and compared these parameters under control, microtubule depolymerized (colchicine treated), and microtubule hyperpolymerized (paclitaxel treated) conditions. From all of these measurements, we found that only the stiffness against shear in the longitudinal plane was modulated by the microtubule cytoskeleton. A simulation model of the myocyte in which microtubules serve as compression-resistant elements successfully reproduced the experimental results. In the complex strain field that living myocytes experience in the body, observed changes in shear stiffness may have a significant influence on the diastolic property of the diseased heart. (Circ Res. 2006;98:81-87.)

Key Words: cytoskeleton ▪ microtubules ▪ cardiomyocyte

In mammalian cells, microtubules and actin filaments constitute the major components of the cytoskeleton and participate in a variety of cellular processes, including organelle transport, cell division, and migration as well as maintenance or alteration of the cell morphology in response to mechanical stimuli transmitted from the surrounding extracellular matrix. However, in the case of postmitotic adult ventricular cardiomyocytes, the functional significance of microtubules may be somewhat different. Their relative content of microtubules (tubulin) is small compared with other types of cells but increases in various disease conditions, such as cardiac hypertrophy or heart failure, in which the heart is subjected to abnormally high loads.

In this context, studies at the tissue (papillary muscle) and cellular levels have focused on the impact of microtubule polymerization on the contractile function of the myocardium, but the results obtained are controversial. The microtubule polymerization observed in hypertrophied hearts was associated with contractile dysfunction and pharmacological disruption of the microtubules by colchicines (COLs) normalized the contractile function. However, in the absence of the preceding hypertrophic proliferation of microtubules, COLs do not improve contractile function. The structural role of the microtubules has also been evaluated by recording the stress–strain relationship of single cardiomyocytes in the longitudinal direction, but the results failed to establish causality for the stiff passive properties observed in the diseased heart.

Studies using magnetic twisting cytometry revealed increased cytoskeletal stiffness and viscosity in hypertrophied myocytes with a high microtubule density, but this methodology did not provide any information regarding the anisotropic properties of these polarized cells or allow evaluation of the reported parameters in the complex strain field that living myocytes experience in the body.

The current study investigated the role of microtubules in the cardiac adaptation process by evaluating the structural properties of single rat cardiomyocytes containing variable amounts of microtubules. In addition to ordinary measurements of the stress–strain relationship in the long axis of each myocyte, we also recorded the transverse stiffness and shear stiffness in both the longitudinal and transverse planes. It was found that microtubules modulate the stiffness of cardiomyocyte only against the shear stress in the longitudinal plane. This result can be taken to indicate that microtubules serve as compression-resistant elements as suggested by the cellular tensegrity model. Simulation study based on this model successfully reproduced the experimental findings.

Materials and Methods

An expanded Materials and Methods section is provided in the online data supplement available at http://circres.ahajournals.org.
Isolation of Cardiomyocytes
Single ventricular myocytes were isolated from 7-week-old female Wistar rats as described previously.1,4 We also studied the myocytes from 10-week-old male cardiomyopathic hamsters (Bio TO-2 strain) and age-matched Syrian golden hamsters (Bio-Breeders Institute, Cambridge, Mass). All the experiments were performed at room temperature. All studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Cell Mechanics Measurements
We characterized the mechanical properties of cardiomyocytes in 3 ways, ie, “tensile stiffness,” “transverse stiffness,” and “shear stiffness.” The tensile stiffness was measured using a cell adhesive carbon fiber–based system as described previously.1,4 Briefly, a rod-shaped quiescent single cardiomyocyte was selected under a microscope and a pair of carbon fibers was attached to both ends using micromanipulators (Figure 1A). One fiber was compliant, whereas the other was thick and rigid and served as a mechanical anchor. The position of the compliant fiber was controlled by a piezoelectric translator (PZT; P-841.40; Physik Instrumente, Karlsruhe, Germany) by a personal computer, and the position of the free end (attached to the cell) was monitored by projecting its image onto a linear photodiode array (S3903; Hamamatsu Photonics, Hamamatsu City, Japan).4 The sarcomere length was simultaneously measured by real-time fast Fourier transform analysis of the striation pattern (IonOptix, Milton, Mass). To measure the tensile stiffness, the cell was stretched at a strain rate of 0.01/sec by pulling the attached carbon fibers, and the tensile stress–strain relationship was obtained.

We used the same experimental setup to measure the transverse stiffness, with some modifications. We attached a latex microsphere (diameter: 5 μm; Polysciences, Warrington, Pa) to the side of the compliant carbon fiber, such that the microsphere could be pushed against the myocyte horizontally (Figure 3A). In each experiment, we selected a myocyte with a rectangular shape, placed it along the sidewall in a glass chamber, and performed an indentation test by moving the compliant fiber (2 μm/sec). Because the area of contact clearly increased during the experiment and this was difficult to quantify, we determined the effective transverse stiffness (Keff) by evaluating the slope of the force-indentation curve as the indentation (δ) approached 0 (mm):

\[ Keff = \frac{\text{applied force}}{\text{indentation}} \]

To evaluate the shear stiffness, we held the myocyte at the bottom coverglass and a small thin glass plate at the top. Small pieces of thin glass plates (thickness: 5 μm; Glass Flakes, REF-160; Nippon Sheet Glass, Tokyo, Japan) were precoated with laminin (Sigma, St Louis, Mo), Under a microscope, a selected single piece of an appropriate size to cover the whole cell was glued to the tip of the thin carbon fiber (Aronalpa; TOAGOSEI, Tokyo, Japan) in the experimental chamber consisting of a Plexiglas frame and a laminin-coated coverglass at the bottom. We gently attached the glass plate to the top surface of the cell (Figure 4A and 4B), and the myocyte was placed either parallel or perpendicular to the direction of the applied shear force. A 10% shear strain was applied by shifting the glass plate connected to a piezoelectric translator via the carbon fiber in 10 seconds in both the longitudinal and transverse plan. All the signals were recorded at 1 kHz by a personal computer (PowerLab/8SP; AD Instruments, Castle Hill, NSW, Australia).

Altering the Polymerization State of Microtubules
To alter the polymerization state of microtubules, COL (Sigma) or paclitaxel (PAC) (Sigma) was added to Tyrode solution and incubated before the mechanics measurements.

Immunocytochemical Procedures
The microtubules were immunocytochemically labeled with a monoclonal antibody against tubulin after fixation, and actin was simul-

Figure 1. Tensile stiffness. A, Tensile stiffness was evaluated by applying a tensile strain via a pair of carbon fibers. The cell was stretched by displacing the attached carbon fibers using a piezoelectric translator. Scale bar=10 μm. B, The tensile stiffness was calculated from the obtained stress–strain relationship (6.02±0.83 mN/mm² at 5% strain and 12.81±0.73 mN/mm² at 10% strain for CTRL myocytes, n=8). Scale bar=10 μm. The stiffness remains unchanged after treatment with COL (6.07±0.44 mN/mm² at 5% and 13.71±0.45 mN/mm² at 10%, n=10) or PAC (6.04±0.54 mN/mm² at 5% and 14.18±1.36 mN/mm² at 10%, n=10). P=0.99 for group comparisons by ANOVA.

Data Analysis
Results are expressed as the mean±SEM. The statistical significance of the microtubule density for each mechanical property was assessed by ANOVA. If statistically significant differences were discovered, pairwise comparisons (Student’s t test) were performed. A probability value of less than 0.05 was considered statistically significant.

Simulation Model
Microtubules, cytoskeletal actin filaments, and desmin filaments (intermediate filaments) were modeled by truss elements (no. 21540), the material properties of which were estimated by either applying bending force to microtubules18,21,22 or recording thermally induced shape fluctuations (statistical mechanical model).22,23 From the reported values ranging from 100 MPa to 1.2 GPa, we adopted an intermediate value of 500 MPa, which corresponds to a persistence length of 2200 μm. Myofibrils were modeled as solid elements (no. 21540), the material properties of which were characterized using the anisotropic hyperelasticity proposed by Humphrey et al for cardiac muscle tissue.24

Results
Mean sarcomere length of studied rat cardiomyocytes was 1.93±0.08 μm (n=8) before the strain was applied. When tensile stress was applied to a myocyte by pulling a pair of carbon fibers attached to both ends of the cell (Figure 1A), the simultaneously measured sarcomere length changed proportionally to the segment length, demonstrating that the stress was distributed homogeneous along the myocyte. From the stress–strain relationship obtained, the average stiffness value for control (CTRL) myocytes (n=8) under tensile stress was determined to be 6.02±0.80 mN/mm² at 5% strain and 12.81±0.73 mN/mm² at 10% strain (Figure 1B). Visualization of the microtubule density in CTRL myocytes by immunolabeling with an anti-β-tubulin antibody (red) revealed a fine network spread over the entire cell surrounding myofibrils (blue), with occasional formation of loop-type structures (Figure 2A). After COL treatment (1 μmol/L, 60
minutes), the mesh-like microtubule network had almost disappeared, whereas the number of microtubules apposed to myofibrils increased significantly after PAC treatment (10 μmol/L, 3 hour) (Figure 2A). These tendencies were confirmed by quantification of the red-light intensities in confocal images normalized to the cell area (Figure 2B). We also noted that these dynamic changes occurred preferentially in the longitudinal microtubules, consistent with previous immunolabeling light and electron microscopic studies.25 These drug-induced changes in the microtubule density did not have any effect on the tensile stiffness of the myocytes (Figure 1B).

The transverse stiffness (K eff) was 11.6±1.6 nN/μm for CTRL myocytes (n=9). K eff tended to change in parallel with the microtubule density (Figure 3B), but the difference did not reach statistical significance (P=0.19 for group comparisons by ANOVA).

We also evaluated the shear stiffness (shear stress/shear strain) in 2 directions, ie, the longitudinal and transverse planes, using a novel technique involving a small glass plate coupled with a carbon fiber (Figure 4A and 4B). The myocyte was held between the bottom coverglass and a small thin glass plate attached onto the top surface. We applied shear stress by shifting the top glass plate connected to a piezoelectric translator via the carbon fiber. Fluorescent staining with a voltage-sensitive indicator (Di2-ANEPEQ) facilitated visualization of the area in contact with the glass plate, and we confirmed that this contact area did not change appreciably during the shear deformation. We confined our analysis to small deformations (linear range) by evaluating the slope at the origin. The shear stiffness values thus obtained for the longitudinal plane (4.57±0.20 kPa, n=15) was nearly double that for the transverse plane (2.94±0.27 kPa, n=13) in CTRL myocytes at 10% strain (Figure 4C and 4D). As in the case of the tensile stiffness, the shear stiffness in the transverse plane did not change significantly by the drug interventions (Figure 4D). However, in the longitudinal plane, hyperpolymerization induced by PAC treatment caused increased the shear stiffness by approximately 2-fold, whereas COL treatment decreased the value by approximately 50% (Figure 4C).

In some myocytes, we evaluated the contribution of cross-bridge formation by repeating these measurements in the calcium-free (Ca 0 mmol/L, EGTA 0.4 mmol/L) solution with 20 mmol/L of butane-dione monoxide (BDM). Inhibition of cross-bridge formation decreased the tensile stiffness.

Figure 2. Alterations of microtubule polymerization. A, Immunolabeling of CTRL, COL-treated, and PAC-treated cells with an anti–β-tubulin antibody (red) and actin stain (blue) by rhodamine phalloidin A fine tubulin network spread over the entire cell, with occasional formation of loop-like structures, and perinuclear distribution are observed in CTRL myocytes. After COL treatment, the mesh-like microtubule network almost disappears, whereas PAC treatment significantly increases the number of longitudinal microtubules apposed to the myofibrils. B, These tendencies are confirmed by quantification of the light intensities of the β-tubulin fluorescence (red) in the confocal images normalized to the cell area. COL: n=9; CTRL: n=9; PAC: n=10 cells. P=0.00 for group comparisons by ANOVA.

Figure 3. Transverse stiffness. A, Transverse stiffness was evaluated by the indentation test. The cell surface was indented transversely using a small microsphere attached to the carbon fiber. Scale bar=10 μm. B, The transverse stiffness was defined as the effective stiffness (K eff) by evaluating the slope of the force-indentation curve at the origin (11.6±1.6 nN/μm for CTRL myocytes, n=9). The transverse stiffness tends to change with the microtubule density after treatment with COL (10.2±1.5 nN/μm, n=10) or PAC (14.7±2.0 nN/μm, n=8), but the differences did not reach statistical significance (P=0.19 for group comparisons by ANOVA).

Figure 4. Shear stiffness. A and B, Shear stiffness was evaluated by applying shear stress in both the longitudinal (A) and transverse (B) planes while holding a myocyte between horizontally arranged glass plates. The shear stress was applied by shifting the top glass plate connected to a piezoelectric translator via the carbon fiber. Scale bars=10 μm. C, The shear stiffness (shear stress/shear strain) in longitudinal planes. Shear stiffness of the CTRL myocytes (4.57±0.20 kPa, n=15) increased after treatment with PAC (7.21±0.56 kPa, n=16) and decreased after treatment with COL (2.70±0.14 kPa, n=16) in the long-axis (fiber) direction (P=0.00 by ANOVA). D, The shear stiffness in transverse planes remains unchanged by the drug interventions (CTRL: 2.94±0.27 kPa, n=12; COL: 3.37±0.21 kPa, n=15; PAC: 3.66±0.25 kPa, n=15) (P=0.13 by ANOVA).
by 27% at 10% strain (Figure 5A). On the other hand, neither transverse stiffness (Figure 5B) nor shear stiffness in both longitudinal and transverse planes was altered by crossbridge inhibition (Figure 5C).

Because a previous study showed the effect of microtubules proliferation on the viscosity of the myocardial tissue in response to the tensile deformation, we also evaluated the viscous properties of cardiomyocyte by applying sinusoidal strain of varying frequencies. The elastic (storage) and viscous (loss) components of the stress/strain modulus were estimated using Fourier transform at the frequency between 1 and 10 Hz (corresponding to the strain rate of 0.1 to 1 sec−1) (Figure 6A and 6B). Whereas the elastic moduli did not change appreciably over the examined frequency range, the viscous moduli were dependent on the strain rate. The slope of the relation between the frequency and the viscous modulus was significantly increased by PAC treatment (COL: 9.4 ± 0.5 mN/mm² per second; CTRL: 11.0 ± 0.8 mN/mm² per second; PAC: 14.8 ± 1.1 mN/mm² per second; n = 6 for each, P < 0.05 PAC vs COL and CTRL) (Figure 6B). All of these results were consistent with the previous report.

In the case of shear in the longitudinal plane, elastic moduli were also independent of the frequency (Figure 6C), but the average value over the tested range (1.0 to 10 Hz corresponding to the shear rate of 0.5 to 5/sec) differed among the 3 groups (COL: 3.4 ± 0.3 kPa; CTRL: 4.8 ± 0.4 kPa; PAC: 8.4 ± 0.6 kPa; n = 6 for each, P < 0.05 for group comparisons by ANOVA). Similar to the tensile stiffness measurement, the viscous moduli were dependent on the shear rate (Figure 5D), and the slope of the relation was significantly greater in PAC-treated myocytes (COL: 0.46 ± 0.01 kPa per second; CTRL: 0.46 ± 0.02 kPa per second; PAC: 0.53 ± 0.013 kPa per second; n = 6 for each, P < 0.05 PAC versus COL and CTRL).

To study the roles of microtubules in pathological conditions, measurements were performed on myocytes from cardiomyopathic (CMP) hamster (Bio-TO2 strain), a well-known hereditary animal model of congestive heart failure using Syrian hamsters as CTRL. The CMP myocytes showed increased level of microtubules proliferation, which was normalized after COL treatment (Figure 7A). Tensile stiffness at 10% strain did not differ between the 2 groups (Figure 7B), but longitudinal shear stiffness was increased in CMP. Furthermore, this increase in shear stiffness was normalized by COL treatment (Figure 7C).
Discussion

In the present study, we measured cellular stiffness as well as stiffness against shear deformation in both the longitudinal and transverse planes. From all of these measurements, we found that changes in the microtubule density induced by drug interventions had marked influences on the shear stiffness in the longitudinal plane. In animal model of heart failure, we also demonstrated that the elevated level of microtubule proliferation in pathological condition was associated with an increase in shear stiffness in longitudinal plane, which was normalized by COL treatment.

Because microtubules have been implicated in many pathological conditions of the heart, such as cardiac hypertrophy, heart failure, and ischemia,5,6,28 many researchers have studied their roles in determining the mechanical properties.11–13,29,30 These studies by applying either stretch or anisosmotic stress to the myocyte or muscle preparations found no significant change in the passive stiffness of the myocardium11,12,29,30 but only found an effect on viscosity of the microtubule proliferation. Our measurement using carbon fiber technique confirmed these findings. Quantitative comparison of the viscosity with previous studies is difficult because various indices of viscosity have been used.12,13 However, in a similar study applying cyclic stretch to the rat papillary muscle,12 Yamamoto et al reported approximately 1.7 fold increase (estimated from their Figure 7) in the slope of the relation between viscous constant (the area of the hysteresis loop) and the strain rate by PAC treatment. The increase in slope identified in this study (∼1.35) is a little smaller, but the use of different index of viscosity may account for this discrepancy.

Although the effect on the tensile stiffness has not yet been definitely identified, a few studies have suggested the mechanical role of microtubules against shear strain. Tagawa et al,13 using magnetic twisting cytometry, showed a 100% increase in the stiffness and a 300% increase in the viscosity after microtubule proliferation induced by pressure overload hypertrophy. Recently, Lammerding et al measured local cell stiffness and reported anisotropy in the material properties of adult mouse cardiomyocytes. Their index of local stiffness differed by a factor of 2 between the longitudinal and transverse directions. We also found that, at the baseline, the cellular shear stiffness was anisotropic in nature (also differed by factor of 2), probably reflecting the preferential distribution of the microtubule density in the longitudinal direction.25 Although the data in these studies13,31 were obtained by applying rotational shear locally using magnetic twisting cytometry, thus not translated into cellular stiffness measured in this study in a straightforward manner, they can be taken to support the present finding.
The change in cross-bridge state induced by BDM did not affect the shear stiffness. This may be a surprising result if the shear stiffness of the intact myocytes measured in this study directly reflects the property of myofibril. However, as Palmer and Ross have shown in isolated rat cardiomyocytes, the lateral coupling between domains of sarcomeres (myofibril) is loose, and these domains slip in response to externally applied force as if they were solid bodies connected by strings. Therefore, we consider that the shear stiffness measured in the intact myocytes mainly reflects the property of cytoskeleton connecting the myofibrils. Similarly, our index of lateral stiffness derived from the initial phase of contact might not probe the small BDM-induced change in myofibrillar stiffness of the resting myocyte.

Why do microtubules modulate only the stiffness against shear strain without changing the tensile stiffness? Gittes et al. measured the flexural rigidity of microtubules and actin filaments to find that the rigidity of microtubules is 3 orders of magnitude greater than that of actin filament. Because the estimated tensile stiffness of a single microtubule was much greater than that of the longitudinal stiffness of the cell, they concluded that, to accommodate strain, microtubules cannot be continuous throughout the length of the cell and that sliding must occur between the filaments. Similar reasoning can be applied to the cardiomycocyte in which microtubule structure has no effect on the tensile stiffness but does affect the viscosity. On the other hand, to modulate the stiffness against shear applied either locally or globally (in the present study), microtubule cytoskeleton must be linked, at least weakly, and anchored to the sarcolemma. We considered that crosslinking with other compliant cytoskeletal structure, eg, actin filament, microtubules can give such mechanical properties to the cardiomycocytes. That is, tensile strain is absorbed by the compliant actin network and the microtubules serve as beams to resist compression when shear stress was applied to the myocytes. The basic idea was similar to the cellular tensegrity model, in which compression-resistant elements (microtubules) support the cell against compression generated by the surrounding tensed cable network to form a structure for mechanotransduction.

We developed a simulation model to evaluate this hypothesis. We used the finite element method to model myofilaments, desmin intermediate filaments, cytoskeletal actin filaments, and microtubules as distinct structures with their respective material properties reported in the literature (supplemental Table). Because the constitutive equation of the myofibril is not available currently, we modified and used the constitutive equation of the myocardial tissue proposed by Humphrey et al. Titin, the major determinant of the passive tensile stiffness of cardiac myocyte at shorter sarcomere length, was not modeled as a distinct element, but included in the myofibril. In addition, the following assumptions were made: (1) actin only bears tension and cannot resist compression; (2) microtubules are elastic and preferentially orientated in the longitudinal direction; (3) actin filaments and microtubules are connected to form the cytoskeleton and are anchored to the sarcolemma (outermost elements) in a discrete fashion; and (4) myofilaments are interconnected transversely by desmin intermediate filaments at the Z-line (Figure 8A and 8B). Because we applied a prestretch (1%) to the actin filaments, all the microtubules were in a compressed state under the control condition (coded in blue to green in Figure 8C). Next, we simulated the effects of tensile stress and shear stress using this model. When we applied a stretch (5%) to the cell, the microtubules became rearranged, but no change in the strain status of the microtubules was observed (Figure 8D). On the other hand, application of a shear (10%) induced high compression of some of the microtubules (green to red in Figure 8E). In accordance with the experimental results, in response to a 70% reduction in the number of microtubules (181 to 52), tensile stiffness (T) and shear stiffness in the transverse plane (S(T)) did not change appreciably, whereas the shear stiffness in the longitudinal plane (S(L)) clearly decreased (Figure 6F). Furthermore, we repeated the calculations under different conditions. (1) To examine whether prestress affect these parameters, we repeated the calculation under the condition of zero prestress. (2) Young’s modulus of microtubule was raised to 1.2 GPa corresponding to a persistence length of 5200 μm. The changes in the final result were modest in both cases (shown in the online data supplement).

Shear deformation of the cardiac tissue has seldom attracted the interest of researchers, probably because the muscle has been regarded as a linear force generator from the conventional physiological point of view. In reality, however, each cardiomyocyte being stretched and contracted in the complex force field of the ventricular wall undergoes significant shear deformation. Oomens et al. measured the 3D strain in the isolated arrested canine left ventricle and found that the shear strain reached 0.05 to 0.1 at the endocardium when a 15 mm Hg of intraventricular pressure was applied. Dokos et al measured the shear properties of passive myocardial tissue to find highly anisotropic nature of myocardium reflecting the alignment of myocytes as well as their laminar structure. Also in beating human left ventricle, release of shear deformation has been demonstrated to play a critical role in relaxation. Furthermore, anisotropy in the shear stiffness has also been suggested to play an important role in cardiac function. The present data clearly showed the importance of microtubules in determining such mechanical properties and could establish a link between the constitutive properties of each myocyte and the whole ventricle. In addition, shear stress may also serve to transmit mechanical signals to the nucleus via the microtubule network during the development of cardiac hypertrophy.

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References

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Supplemental Documentation

Results

The effect of BDM on transverse stiffness

We did not find a significant effect of crossbridge formation on the transverse stiffness. This result may contradict the study using an atomic force microscope demonstrating the dependence of the transverse stiffness of a single myofibril on the crossbridge formation\(^1\). However, we should be careful in interpreting their data. Firstly, although they identified large difference in stiffness between rigor and relaxed states, the difference between active (pCa 4.5) and relaxed (pCa 8) states were relatively small. We could reasonably expect that the effect of BDM applied to the resting myocyte would be much smaller. Secondly, in the current study on intact myocytes, the cantilever might not probe the myofibril directly especially during the initial phase of contact. Because our index of transverse stiffness was calculated from this initial phase of recording, the effect of BDM could have been masked. Based on such reasoning, we repeated the indentation test of larger amplitude (to probe the myofibril) with 20mM of Ca\(^{2+}\) (to increase the number of attached crossbridges) in the absence and presence of BDM (N=10 cells for each condition). As shown in the Supplementary Figure 1, indentation force became significantly larger with 20mM Ca\(^{2+}\) for large indentation (2 and 3 µm) compared with 20mM Ca\(^{2+}\) with 10mM BDM and 1.1mM Ca\(^{2+}\). Although these results should be interpreted with cautions because of the ambiguity in contact area, the effect of BDM became clear with larger indentation for high Ca\(^{2+}\) condition.

Effects of prestretch to actin filament and Young’s modulus of microtubule on simulated cell stiffness

In the tensegrity model, tensile stress (prestress) carried by microfilament (actin filament) is balanced by the compression-resistant element (microtubule) to form self-stabilizing structure and the prestress is not necessarily related to the externally applied stretch. Because, however, it has also been suggested that the prestress can modulate the behavior of the cell\(^2\), we repeated the simulation in the absence of pre-stretch (0%). Within this small range of variation in prestretch, the final results were affected only modestly (Supplementary Figure 2).

We also evaluated the change in Young’s modulus by simulation. As for the parameters characterizing microtubules, many reports have been published using various experimental approaches. Those are either bending microtubules using optical tweezers\(^3\), hydrodynamic flow\(^4\) or the atomic force microscope, or by recording thermally induced shape fluctuations (statistical mechanics model)\(^4,5\). The reported values for Young’s modulus differ among the studies ranging from 100 MPa to 1.2GPa. In this study we adopted the intermediate value of 500 MPa which corresponds to a persistence length of 2200µm\(^4\). To see how the variation in Young’s modulus affect the cellular stiffness, we repeated the simulation using a larger Young’s modulus (1.2 GPa corresponding to a persistence length of 5200µm\(^7\). As shown in figure 8-F (500MPa), and supplementary figure 3 (1.2GPa), the difference in longitudinal shear stiffness between control and colchicine was similar in both conditions.
Expanded material and methods

Isolation of cardiomyocytes

Single ventricular myocytes were obtained from 7-wk-old female Wistar rats. We also studied the myocytes from 10-wk-old male cardiomyopathic hamsters (Bio TO-2 strain) and age-matched Syrian golden hamsters (Bio-Breeders Institute, Cambridge, MA). The heart was quickly removed under phenobarbital anesthesia and cells were enzymatically isolated using a modified dispersion technique as described previously. After isolation, the extracellular calcium concentration of the Tyrode solution was gradually increased to 1.1 mM, and the myocytes were transferred to the experimental chamber. All the experiments were performed at room temperature. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Cell mechanics measurements

We characterized the mechanical properties of cardiomyocytes in three ways, i.e., “tensile stiffness”, “transverse stiffness” and “shear stiffness”.

The “tensile stiffness” was measured using a cell adhesive carbon fiber-based system as described previously. Briefly, a rod-shaped quiescent single cardiomyocyte was selected under a microscope and a pair of carbon fibers was attached to both ends using micromanipulators (Fig. 1-A). One fiber was compliant (diameter: 7µm; length: 1-1.2mm; stiffness: 80-200 nN/µm), while the other was thick and rigid (diameter: 30µm; length: ~1mm; stiffness: >1,000 nN/µm) and served as a mechanical anchor. The position of the compliant fiber was controlled by a piezoelectric translator (PZT; P-841.40; Physik Instrumente, Germany) fixed to it, via appropriate command signals generated by a PC. The position of the free end (attached to the cell) was monitored by projecting its image onto a linear photodiode array (S3903; Hamamatsu Photonics, Japan). To determine the force applied to the cell, the strain of the fiber (distance between the cell end and the PZT end) was multiplied by the fiber stiffness. The sarcomere length was simultaneously measured by real-time FFT of the striation pattern (IonOptix, Milton, MA, USA). To measure the “tensile stiffness”, the cell was stretched at a strain rate of 0.01/sec by pulling the attached carbon fibers, and the tensile stress-strain relationship was obtained.

We used the same experimental set-up to measure the “transverse stiffness”, with some modifications. To visualize and measure both the displacement of the cantilever and the cell surface using an optical microscope, we performed a sideways indentation test. We attached a latex microsphere (diameter: 5 µm; Polysciences, PA, USA) to the side of the tip of the compliant carbon fiber, such that the microsphere could be pushed against the myocyte horizontally (Fig. 3-A). In each experiment, we selected a myocyte with a rectangular shape and placed it along the sidewall in a glass chamber (width: 5mm; depth: 5mm; height: 300µm) custom-made by a high precision molding technique (Nalux, Japan). Next, we performed an indentation test by moving the compliant fiber at a constant velocity (2µm/sec). The position of the microsphere (indentation) and the strain were determined in a similar manner to the tensile stiffness measurements. Since the area of contact clearly increased during the experiment and this was difficult to quantify, we determined the effective transverse stiffness (Keff) by evaluating the slope of the force-indentation curve as the indentation (δ) approached zero.
To evaluate the “shear stiffness”, we held the myocyte between the bottom coverglass and a small thin glass plate at the top. Small pieces of thin glass plates (dimensions: 100-150 x 100-150 µm; thickness: 5µm; Glass Flakes, REF-160; Nippon Sheet Glass, Japan) were pre-coated with laminin (Sigma, MO, USA). Under a microscope, we selected a single piece of an appropriate size to cover the whole cell and glued it to the tip of the thin carbon fiber (aronalpha; TOAGOSEI, Japan). Through this procedure, the glass flake was set parallel to the stage of the microscope. Next, the experimental chamber consisting of a plexiglas frame and a laminin-coated coverglass at the bottom was placed on the microscope stage. Among the myocytes introduced into the experimental chamber, we selected a rod-shaped cell with a relatively flat appearance, and gently attached the glass plate to the top surface of the cell (Figs. 4-A and 4-B). To determine the shear stiffness in both the longitudinal and transverse planes, the myocyte was placed either parallel or perpendicular to the direction of the applied shear force. A 10% strain was applied by shifting the glass flake connected to a piezoelectric translator via the carbon fiber in 10 seconds. The cell length and width were measured from video images, and the height was estimated by focusing the image while adjusting the z-axis of the microscope. Furthermore, to clearly visualize and measure the cell contact area covered by the glass flake, we loaded the myocyte with a voltage-sensitive dye (Di2-ANEPEQ, Molecular Probes, CA, USA). Under phase-contrast microscopy, the contact area between the glass flake and the cell membrane was not clearly identified. Voltage-sensitive dye has the following advantages to solve this problem. 1) It binds to the cell membrane but exhibits low fluorescence in aqueous environments in the cytosol. 2) It emits light of fairly high intensity even at the resting potential of the unstimulated myocytes. Finally, because the portion cell membrane attached to the glass flake became flat and positioned in parallel to the stage, the whole area and its margin were visible clearly with fluorescence when we focused on the glass flake. Fluorescent images were observed and recorded on the video-tape and digitized off-line. From these images, we calculated the contact area with the resolution of 200nm per pixel, and confirmed that the contact area was constant while the deformation was applied. All the signals were recorded at 1kHz by an A-D converter connected to a PC (PowerLab/8SP; AD Instruments, Australia). Based on these measurements, we calculated the stress (force/unit area) and strain according to the same principles used for the stiffness measurements.

**Altering the polymerization state of microtubules**

To study the effects of microtubule depolymerization, colchicine (Sigma, MO, USA) at a final concentration of 1µM was added to the Tyrode solution and incubated for 1h. To assess the effects of microtubule hyper-polymerization, myocytes were incubated with 10µM of paclitaxel (Sigma, MO, USA) for 3h. In both cases, the mechanics measurements were performed after the incubation.

**Immunocytochemical procedures**

To confirm and quantify the polymerization state, the microtubules were labeled with a monoclonal antibody against β-tubulin (Zymed Laboratories, CA, USA) and an FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, PA, USA) after fixation as described previously. Actin was simultaneously stained with rhodamine phalloidin (Molecular Probes, CA, USA). The cells were observed
using a laser confocal microscope (CSU21; Yokogawa-denki, Japan) using PlanApo x 100/1.4 oil objective, and the mean fluorescence intensity normalized by the section area of beta-tubulin was determined using IPLab software v3.6.4. (Scanalytics, VA, USA).

**Data analysis**

Results are expressed as the mean ± SEM. The statistical significance of the microtubule density for each mechanical property was assessed by analysis of variance (ANOVA). If statistically significant differences were discovered, pairwise comparisons (Student’s *t*-test) were performed. A *p* value of less than 0.05 was considered statistically significant.

**Simulation model**

Microtubules, cytoskeletal actin filaments and desmin filaments (intermediate filaments) were modeled by truss elements (number 1874) using the Young’s moduli and geometrical parameters listed in Supplementary Table 1. Young’s modulus of microtubule has been estimated by either applying bending force to microtubules, or recording thermally induced shape fluctuations (statistical mechanics model). From the reported values ranging from 100 MPa to 1.2 GPa, we adopted an intermediate value of 500 MPa, which corresponds to a persistence length of 2200 µm. Myofibrils were modeled as solid elements (number 21540), the material properties of which were characterized using the anisotropic hyper-elasticity proposed by Humphrey et al. for cardiac muscle tissue. The constitutive equations and their parameters (*c*\textsubscript{i}) are as follows:

\[
W = c_1(\alpha - 1)^2 + c_2(\alpha - 1)^3 + c_3(I_1 - 3) + c_4(I_1 - 3)(\alpha - 1) + c_5(I_1 - 3)^2
\]

\[
I_1 = trC
\]

\[
\alpha^2 = N \cdot C \cdot N
\]

where *W* is the strain energy function, *C* is the right Cauchy-Green deformation tensor, and *N* is a unit vector defining the preferred direction of the material in the undeformed configuration. Parameter values (*c_1* = 6.05783, *c_2* = 19.1288, *c_3* = 0.602244, *c_4* = -0.989456, *c_5* = 8.60661) were adjusted to reproduce the experimental results.

Utilizing the symmetric nature of the problem, we only modeled half a myocyte to reduce the computational time. The total number of degrees of freedom was 154466. Using parallel computing with ten Pentium4 processors (3.2 GHz), 2.5 min were required to calculate each protocol.
### Supplementary Table 1

Parameters for the simulation model

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Reference


Figure legends

**Online Figure 1** The relation between indentation and indentation force. Indentation force required to make small indentation (1µm) did not differ among 1.1mM Ca$^{2+}$ (open triangle), 20mM Ca$^{2+}$ + 20mM BDM (open circle), and 20mM Ca$^{2+}$ (closed circle). At larger indentation, indentation force became significantly larger for 20mM Ca$^{2+}$. *: p<0.05 between 20mM Ca$^{2+}$ + 20mM BDM and 20mM Ca$^{2+}$, †: p<0.05 between 1.1mM Ca$^{2+}$ and 20mM Ca$^{2+}$.

**Online Figure 2** The effect of pre-stretch on simulated cell stiffness. **A**: Tensile stiffness at 5% strain. **B**: Shear stiffness in longitudinal (L) and transverse (T) planes at 10% strain. In each panel the effect of reduction in the number of microtubules was evaluated in the absence (0%) and presence (1%) of pre-stretch. Black bars: control (CTRL) condition; white bars: reduced number of microtubules mimicking colchicine (COL) treatment.

**Online Figure 3** The effect of Young’s modulus of microtubule on cell stiffness. The simulation was repeated using a larger Young’s modulus (1.2GPa). **A**: Tensile stiffness at 5% strain. **B**: Shear stiffness in longitudinal (L) plane and transverse (T) plane at strain 10%. Black bars: control (CTRL) condition; white bars: reduced number of microtubules mimicking colchicine (COL) treatment.